The effect of administration of double stranded MicroRNA-210 on acceleration of Achilles tendon healing in a rat model

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Abstract

Background Achilles tendons heal slower than other tissues, therefore requiring the development of a strategy for accelerating the process. Vascular supply plays an important role in primary tendon healing, especially during the early healing phase. MicroRNA (miR)-210 has been reported as being crucial for angiogenesis, which is a key factor of tissue repair. We report herein that local injection of synthetic miR-210 into the injured Achilles tendon of a rat accelerated healing of the tendon.

Methods Achilles tendons were transected and repaired via the Kessler suture technique in Sprague–Dawley rats. Then, double stranded (ds) miR-210 was injected into the repaired sites. The control group was injected with non-functional dsRNA. At 2, 6 and 12 weeks, histological evaluations were performed. At two and six weeks, mechanical testing and angiogenesis were evaluated. Gene expression analysis using real-time polymerase chain reaction (PCR) and immunohistochemistry were performed at two weeks.

Result At two and six weeks, regular dense collagen tissue in the miR-210 group was observed and the diameter of collagen fiber in the miR-210 group was significantly higher than in the control. At two weeks, the ultimate failure load was significantly higher than in the control group, and expression of VEGF, FGF2 and type I collagen was upregulated. Abundant vessels in the miR-210 group were observed at two weeks, but there was no significant difference in vessel numbers between the two groups at six weeks. At 12 weeks, repaired Achilles tendons in the miR-210 group consisted of parallel and dense fibers, whereas wavy and loose fibers were still observed in the control group.

Conclusion The current study showed that single local injection of synthetic miR-210 promotes Achilles tendon healing in the early phase.

Introduction

Achilles tendon injury is a very common injury in the orthopaedic field, usually due to trauma or sport injury [1]. However, its optimal treatment is still debatable in spite of the high rupture incidence. Several reports comparing operative treatment to non-operative treatment focused on the rate of re-rupture and complications. Some papers demonstrated that non-operative treatment resulted in a higher incidence of re-rupture [2–4], while no significant difference in the re-rupture rate between non-operative and operative treatment was reported in several papers [5, 6]. The most important aspect of Achilles tendon injury is that they heal at a slower rate than other connective tissues because of the reduced cellular component of the tendon tissue. Therefore, various studies have attempted to accelerate Achilles tendon healing using drugs, cytokines and cell therapy [7–9].

MicroRNAs (miRNAs) are small non-coding RNA molecules that are well known as important key players in a variety of biological processes. miRNAs negatively regulate gene expression by inhibiting translation...
or degradation of mRNA (messenger RNA) through the binding the 3' untranslated region (3' UTR) of their target mRNA [10-13]. miRNA plays an important role in the pathogenesis of many human diseases. Therefore, several therapeutic trials regulating endogenous miRNA in vivo have been conducted [14-16]. miRNA also plays a crucial role in the pathogenesis of disease or trauma in locomotor organs [17-21]. As we known, vascular supply in tendons is very limited and is significantly reduced after injury or rupture. We believe angiogenesis activation of endothelial cells may play an important role in promoting and regulating other biological events that can increase vascular permeability in the tendon healing process. MiRNA (miR)-210 is capable of inducing angiogenesis by targeting ephrin-A3 (EFNA3) [22]. miR-210 is upregulated in response to hypoxia, which affects cell survival, migration, and differentiation. Moreover, overexpression of miR-210 enhances the formation of capillary-like structures [23, 24]. Therefore, our hypothesis is that administration of miR-210 can enhance tendon healing via acceleration of angiogenesis in an early phase, which will subsequently promote maturation of the Achilles tendon. The purpose of this study is to examine the therapeutic effect of a single administration of miR-210 into the ruptured Achilles tendon in a rat model.

Materials and methods

Animal model

This study was reviewed and approving by the Ethics Committee for Experimental Animals of Hiroshima University and the animals were treated according to the guidelines of the Institutional Animal Care and Use Committee.

Male 12-week-old Sprague-Dawley rats weighing 200-250 g were used in this study. The rats were housed under standard conditions at the Laboratory Animal Centre at the Hiroshima University. They were fed a standard commercial diet and water. The injured Achilles tendon model was created according to the previous report [7]. Under general anesthesia, a 3-cm longitudinal skin incision was made over the Achilles tendon of the right leg. The surrounding fascia was cut longitudinally, and the Achilles tendon and plantaris tendon were exposed. The Achilles tendon was cut transversely 5 mm from its calcaneal insertion. The Achilles tendon was repaired with 7-0 monofilament nylon sutures using a modified Kessler-type suture technique. The plantaris tendon was preserved. The wound was closed with 5-0 monofilament nylon sutures. After surgery, 30 μl of osteocollagen (Koken, Tokyo, Japan)/double stranded (ds) RNA (osteocollagen 15 μl; dsRNA 20 μg) was injected percutaneously into the repaired tendon (Fig. 1). Ds miR-210 (sequences

66-CUG-UGC-GUG-UGA-CAG-CUG-A-87 and 87-AGC-CCC-UGC-CCA-CCG-CAC-ACU-G-66 labeled with FAM, B-Bridge International) was injected into the repaired tendon site in the miR-210 group, and non-functional ds RNA (dsRNA negative control, B-bridge International; ds Nega) was injected in the control group. The rats without any ds RNA injection after surgery were also evaluated as the non-injection group. The rats were placed in their cages and afforded unrestrained mobility. At 24 h, 3 days, and 2, 6 and 12 weeks after operation all rats were killed by overdose injection of anesthesia. The Achilles tendon was removed with the attached calcaneal bone and dissected from other surrounding tissue and prepared for histological evaluation, gene expression analysis, and mechanical testing. Five rats in each group were used to test tensile strengths, and five other rats were used to test gene expression. The remaining rats in each group were used for histological evaluation.

Histological analysis

At 2, 6 and 12 weeks after surgery, 5 rats in each group at each time point were sacrificed. Then, Achilles tendons were harvested and quickly embedded in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC, USA), snap-frozen in liquid nitrogen. Sections 6 μm thick were prepared and Masson trichome staining was performed. The specimens were examined in a random order with a microscope and two observers (MAU, TS) evaluated the specimens according to an arbitrary scoring system from 1 to 5, where 1 represented an immature loose calculus and 5 represented a dense organized fibrous tissue with
mostly parallel fibers, according to the previous report [21]. Five microscopic areas from each specimen at ×200 magnification were randomly chosen and the diameter of newly formed collagen fiber was measured by the two authors (MAU, TN) at two and six weeks using a digital microscope (BZ-9000, Keyence, Japan).

To evaluate the distribution of the injected ds miRNA, rats were sacrificed 24 h after injection and 6-μm serial sections were mounted on saline-coated glass slides, air-dried, and fixed with 4.0 % paraformaldehyde at 4 °C for 5 min. Then, 4',6-diamidino-2-phenylindole (DAPI) solution was applied for 5 min for nuclear staining. FAM-labeled miR-210 in Achilles tendon specimens was detected using fluorescence microscopy.

Immunohistochemistry analysis

For immunohistochemistry staining of Isoclectin B4, VEGF, FGF2 and Collagen type I, specimens were fixed with 4.0 % paraformaldehyde at 4 °C for 5 min. After blocking by horse serum (Vector Laboratories, Burlingame, California), specimens were stained with rabbit polyclonal anti-VEGF or anti basic FGF antibody (Abcam, Cambridge, Massachusetts, concentration 1:100), goat polyclonal anti-collagen type I antibody (Santa Cruz Biotechnology, Santa Cruz, California; concentration 1:50), or fluorescein-labeled GSL I-isoclectinB4 (Vector Laboratories, Burlingame, California; concentration 1:100). The secondary antibodies used were Alexa Fluor 488-conjugated or Alexa Fluor 568-conjugated goat anti-rabbit IgG for VEGF and FGF2, and Alexa 488-conjugated or Alexa 568-conjugated rabbit anti-goat IgG for collagen type I (all from Molecular Probes/Invitrogen, Carlsbad, California). DAPI solution was applied for 5 min to detect nuclear staining. The negative control was prepared in the same manner without the primary antibody. For evaluating newly formed vessels, five microscopic areas (250 × 250 μm) from each specimen at ×200 magnification were randomly chosen. Capillaries were recognized as tubular structures positive for isoclectin B4, and counted by the two authors (MAU, TN).

Mechanical testing

For testing mechanical properties at two and six weeks, a conventional tensile tester (1840 NT/500; AIKOH Engineering, Osaka, Japan) was used. All of the extra soft tissue and hard tissue was removed from the calcaneal or Achilles tendon complex by blunt and/or sharp dissection. The calcaneus-Achilles tendon complex was fixed between two metal clamps and pulled at a constant speed of 200 mm/min vertically until rupture. The maximum load recorded was recognized as the ultimate tensile strength. In addition, the calcaneus-Achilles tendon complex from other normal rats were measured as normal tendons.

Quantitative real-time PCR

Total RNA was isolated from repaired Achilles tendon site using TRIzol. Complementary DNA was synthesized using "Ready to Go You-Prime First-Strand Beads" (GE Healthcare, Chalfont, UK) with total RNA (1 μg) and oligo (dT) primers. For miRNA expression analysis, reverse transcriptase reactions of mature miRNAs contained a sample of total RNA, 50 nM of stem-loop RT primer, 10 × RT buffer, 100 mM of each dNTP, 50 U/μl of MultiScribe Reverse Transcriptase, and 20 U/μl of RNase inhibitor. 15-μl reactions were incubated in a thermocycler (Bio-Rad, Hercules, CA). Real-time quantitative PCR was carried out using TaqMan Gene Expression Assay probes for sno-miR-210, snoRNA-135, VEGF, COL1A1, FGF2 and ACTB. The expression levels for each gene were assessed relative to the expression of snoRNA-135 for miR-210, and ACTB for other genes. A threshold cycle (Ct) was observed in the exponential amplification phase, and quantification of relative expression levels was performed using standard curves for target genes and the endogenous control. Geometric means were used to calculate the ∆∆Ct (delta-delta Ct) values and expressed as 2^-∆∆Ct. The value of each control sample was set at one and used to calculate the fold-change of target genes.

Statistical analysis

All results in this study were expressed as the mean ± standard deviation (SD). Comparison among the three groups was done using the Tukey–Kramer post hoc test. A p value of <0.05 was consider statistically significant. All data were calculated and shown as value ±SD.

Results

Administration of ds miR-210 could enhance morphological and functional recovery

Per their gross appearance at two weeks, the Achilles tendon surfaces in the control group were irregular and dille was observed in the repaired site. On the other hand, repaired Achilles tendon surfaces in the miR-210 treated group were smoother and bulkier than in the control group. Histological analyses of Masson trichrome staining demonstrated that the injury site was filled by a dense organized fibrous tissue with mostly parallel fibers in the miR-210 group, while immature loose fibrous tissue was observed in the control and non-injection groups (Fig. 2a). At six weeks,
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Fig. 2 a Masson trichrome staining of repaired site in the non-injection, control and miR-210 groups at two and six weeks. Bars represent 100 μm. b Collagen fiber diameter in all groups at two and six weeks. *p < 0.05. c Distribution of FAM-labeled dsmiR-210 at 24 h after injection. Arrows indicate FAM-labeled ds miR-210. Dotted lines indicate the Achilles tendon outline. *Achilles tendon. Bars represent 100 μm

Fig. 3 Mechanical testing at two and six weeks. *p < 0.05. NS no significant difference

abundant collagen fibers with little fibrous tissue occupied the injured site in the miR-210 group, while the abundant loose fibrous tissue still remained in the control and non-injection groups (Fig. 2a). The histological score of the miR-210 group (4.4 ± 0.2 points) was significantly higher than the control (1.4 ± 0.2 points) and non-injection groups (1.4 ± 0.5) at two weeks, and the histological score in the miR-210 group was significantly higher than that in the control and non-injection groups (non-injection group, 1.8 ± 0.8 points; control group, 1.8 ± 0.8 points; miR-210 group, 4.6 ± 0.5 points) at six weeks. The mean collagen fiber diameter in the miR-210 group was significantly higher than that in the control (non-injection group, 2.4 ± 0.2 μm; control group, 2.5 ± 0.3 μm; miR-210 group, 4.0 ± 0.2 μm) (p < 0.05) at two weeks, and the mean collagen fiber in the miR-210 group was significantly higher than that in the controls (non-injection group, 4.4 ± 0.4 μm; control group, 4.2 ± 0.3 μm; miR-210 group, 6.0 ± 0.3 μm) (p < 0.05) at six weeks (Fig. 2b). FAM-labeled dsmiR-210 was detected in the tendon near the injured site and around the tendon (Fig. 2c). The ultimate failure load of the Achilles tendon in the miR-210 group (25.3 ± 7.3 N) was significantly higher than that in the non-injection group (13.2 ± 1.7 N) and control group (14.6 ± 1.7 N) at two weeks after local injection (p < 0.05) (Fig. 3). At six weeks, there was no significant difference in the ultimate failure load between both groups (p = 0.453), but the ultimate failure load in the miR-210 group (73.0 ± 22.4 N) was likely to be higher
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than that in the non-injection (65.9 ±17.3 N) and control groups (70.4 ±22.4 N). In all groups, the failure site was the repaired site in all specimens. These findings indicate that single local injection of ds miR-210 could enhance morphological and functional recovery at two weeks.

To examine the effect of miR-210 administered on angiogenesis during an early phase, immunohistochemistry of isolec tin B4 was performed. In the miR-210 group, abundant vessels in the repaired site were observed while there were few newly-formed vessels in the non-injection and control groups (Fig. 4a). The capillary density in miR-210 group was significantly higher than that in the non-injection and control groups (non-injection group, 31.4 ±5.3; control group, 32.6 ±8.3; miR-210 group, 72.1 ±12.7 capillary profiles per mm², p < 0.05). Although miR-210 promotes healing by angiogenesis during the early phase, there was no significant difference in the number of blood vessels between all groups (non-injection group, 75.5 ±4.3; control group, 76.2 ±7.6; miR-210 group, 84.5 ±15.4 capillary profiles per mm²) at six weeks, indicating blood vessel retraction during a subsequent remodeling phase, during tendon maturation by miR-210 injection (Fig. 4b).

Gene expression analysis

Firstly, the expression level of miR-210 at three days after injection was evaluated by real-time PCR. The expression of mature mno-miR-210 was significantly upregulated in the miR-210 group compared to the non-injection and control groups (p < 0.05) (Fig. 5a). VEGF, which is an important factor of angiogenesis in the healing process, was upregulated in the miR-210 group as compared to the non-injection and control groups (Fig. 5b). Also, expression of FGF2 and Col1α1 in the miR-210 group was significantly higher than in the non-injection and control groups (Fig. 5c, d). The immunohistochemistry of VEGF, FGF2 and type I
Fig. 5 Gene expression analysis of miR-210, VEGFA, FGF2 and Coll1a1 by real-time PCR three days after injection. *p < 0.05

collagen two weeks after injection was more expressed at the injured sites in the miR-210 group as compared to the control group (Fig. 6).

Administration of ds miR-210 can promote maturation of the Achilles tendon

At 12 weeks, repaired Achilles tendons in the miR-210 group consisted of parallel and dense fibers, whereas the control group still exhibited wavy and loose fibers, although newly-formed collagen fibers in the Achilles tendon in both groups were remodeled and had matured (Fig. 6).

Discussion

Vascular supply plays an important role in primary tendon healing [26, 27]. Tendon healing undergoes three phase, including an inflammatory phase, a proliferative phase, and a remodeling phase [28]. During these phases, especially at the early stage, blood supply provides not only cell infiltration, including inflammatory cells and fibroblasts, but also supplies reparative factors such as multiple growth factors, oxygen, and so on. It is expected that the promotion of neo-vascularization accelerates tendon healing by up-regulation of endogenous reparative factors. Growth factors such as VEGF, TGF-β1, PDGF, and FGF2 also have multiple significant roles in tendon healing [29]. During the tendon healing process, each growth factor has a crucial function at different stages and work in cooperation with other growth factors during such. Several studies have attempted to improve the primary tendon healing process by applying exogenous growth factor in animal models, demonstrating enhanced tendon healing [7]. In most of these studies, however, the application of a single growth factor was conducted. The application of a single exogenous growth factor
might not be enough to further improve and accelerate physiological tendon healing. Notably, one miRNA has a hundred target genes estimated by computational analysis, meaning miRNA can regulate many gene networks simultaneously. This could be one of the advantages of miRNA therapy. It has been reported that miRNA-210 plays a crucial role in angiogenesis in response to hypoxia via targeting Ephrin-A3, E2F3, NPTX1, RAD52, ACVR1B, MNT, CASP8AP2, FGFRL1, and HOXA-1 [22–24]. Hu et al. [28] demonstrated that local injection of ds miR-210 into the myocardium improves cardiac function through angiogenesis, inhibiting apoptosis. Shoji et al. [16] reported that intra-articular injection of ds miR-210 for partial rupture of the anterior cruciate ligament accelerated ligament healing in a rat model. In the orthopaedic field, reports of therapeutic trials in vivo using several miRNAs have increased. It has been reported that local injection of muscle-specific miRNAs accelerates muscle regeneration in a rat muscle injury model [21]. Systemic injection of miR-146a ameliorated joint destruction in a collagen-induced mouse model. Masson trichrome staining revealed the collagen fibers in the miR-210 treated group become more aligned and denser than that in the control group at two weeks. In immunohistochemistry analysis of type I collagen, the synthesis of type I collagen in the miR-210-treated group was higher than that in the control group, which usually begins at about six weeks, during the remodeling phase. In previous reports, TGF-β1 promoted collagen synthesis in repaired Achilles tendons in rats, subsequently increasing the tensile strength at two and four weeks [30]. Our mechanical testing results showed that administering ds miR-210 increased the tensile strength in the repaired Achilles tendon at two weeks after surgery. Improvement during the early phase of primary tendon healing has become more and more important since several clinical studies have indicated the benefit of early mobilization after surgery. The most significant result of the present study, therefore, is that local injection of ds miR-210 into an injured Achilles tendon can give rise to higher tensile strength and aid the histological healing process of advanced tissue during the early phase, leading to more mature Achilles tendon healing at six weeks (Fig. 7).

Tendons are largely avascular. The in-growth of vessels contributes to tendon repair during the early phase but over-vasculature in the tendon may cause chronic tendinopathy. In our results, abundant blood vessels at the repair site in the miR-210 group were observed, but the blood vessels retracted during the subsequent remodeling phase,
indicating that single local injection of ds miR-210 did not induce lasting over-vasculature, resulting in impaired tendon function. Blood vessel retraction during the latter phase may be due to the acceleration of remodeling induced by miR-210 injection.

There are several limitations to this study. First, it is unclear what kind of cells miR-210 affect. FAM-labeled miR-210 was detected in the tendon and surrounding tissue. It has been reported that miR-210 can function in the endothelial cells. Therefore, miR-210 might be taken up in the endothelial cells around the repaired tendon site, and there was a possibility that miR-210 might work in fibroblasts in the tendon. Second, the precise molecular mechanism by which miR-210 enhances Achilles tendon healing has not been elucidated. In our study, VEGF and FGF2, which play important roles in angiogenesis, were upregulated by miR-210, and the acceleration of tendon healing might enhance collagen type I synthesis. However, its process, including changing the expression pattern of miR-210 target genes, could not be elucidated. miRNAs have hundreds of target gene, as determined by computational analysis. Therefore, it is challenging to elucidate the mechanism completely. These results suggest that administrated miR-210, which was taken up or which upregulated the expression of the endogenous miR-210 in the injured Achilles tendon, could have upregulated VEGF and FGF2 expression, and subsequently induced type I collagen, which is a main functional component of tendons during the early tendon healing phase. Finally, there was a possibility that the non-specific response, such as an unexpected vascular or immune response to the injected synthetic miR-210, could of enhanced Achilles tendon healing. However, we created a non-RNA injection control model and it exhibited the same results of non-specific, functional ds RNA. These data and the previous report of Shoji et al. suggest that miR-210 injection had a specific effect on enhancement of Achilles tendon healing.

In conclusion, our study shows the potential of administering ds miR-210 for the acceleration of tendon repair. It is speculated that miR-210 accelerates neovascularization and, consequently, induces a microenvironment conducive to tendon healing during the early phase. Nevertheless, the details of this mechanism remain unclear. Single local injection of ds miR-210 may become a new therapeutic strategy for promoting tendon healing.

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Conflict of interest The authors declare that they have no conflict of interest.

References

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